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Jane Hubert, Jean-Marc Nuzillard, Jean-Hugues Renault

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Jane Hubert[a]*, Jean-Marc Nuzillard[a], Jean-Hugues Renault[a]

AUTHOR ADDRESS

[a]Institut de Chimie Moléculaire de Reims (UMR CNRS 7312), SFR CAP'SANTE, UFR de Pharmacie, Université de Reims Champagne-Ardenne, Reims, France

*Corresponding author: jane.hubert@univ-reims.fr

ABSTRACT

The development of new drugs will certainly benefit from an ever improving knowledge of the living beings chemistry. However, identification of drugable molecules within the immense biodiversity of forests, soils or oceans still requires considerable investments in technical equipments, time and human resources. An important part of this process is the quick identification of known substances in order to concentrate the efforts on the discovery of new ones. A range of "dereplication" procedures are currently emerging to meet this challenge as key strategies to improve the performance of natural product screening programs. Initially defined in 1990 as "a process of quickly identifying known chemotypes", dereplication is today a not so univocal concept and has evolved over the last years in different ways. The present review covers all dereplication-related sudies in natural product research from 1990 to 2014. Its writing brought to light five distinct dereplication workflows that can be characterized by the nature of starting materials, by the key analytical technique, and above all by the final objective. Dereplication can be used as an untargeted workflow for the rapid identification of the major compounds whatever their chemical class in a single sample or for the acceleration of bioactivity-guided fractionation procedures. In other cases dereplication is fully integrated in metabolomic studies for the untargeted chemical profiling of natural extract collections or for the targeted identification of a predetermined class of metabolites. Finally a quite distinct dereplication approach mainly based on gene-sequence analyses is frequently used for the taxonomic identification of microbial strains.

KEYWORDS

Dereplication, natural products, metabolomics, drug discovery, taxonomic classification.

Introduction

Living beings provide a unique diversity of secondary metabolites that are still waiting to be investigated and exploited for the discovery of new drugs, cosmetic ingredients, nutraceutics or bio-sourced materials. The performance of natural product screening programs remains however strongly limited by the equipment, labor and time investments required for isolation and structural elucidation of novel metabolites with potent biological properties. Significant advances have been made over the last decades to improve the resolution and sensitivity of analytical methods for the study of small molecules in natural extracts. Very efficient hyphenation systems between separation and spectroscopic instruments now enable the identification of highly complex molecular structures even within complex mixtures of compounds. Despite these efforts, very serious issues, such as the frequent rediscovery of known compounds after time consuming purification and identification steps, the redundant investigation of taxonomically ambiguous microbial strains or the investigation of irrelevant crude materials regarding a selected biological application, still result in a considerable waste of time. Consequently, many pharmaceutical companies have considerably slowed down or even terminated their natural product research activities.

Nevertheless, the search for novel chemical structures within natural resources continues to be a crucial strategy for the discovery of novel therapeutic drugs. Moreover, remembering that more than 300 000 secondary metabolites have been identified so far from the biodiversity, it is obvious that new biological activities of known metabolites can be still put to light . Some of these known metabolites have only been structurally elucidated but never evaluated biologically. Among those that have already been engaged in one or even more specific bioassays, it is certain that a wide majority have not yet revealed all their biological potential, just because it remains impossible to test every compound on every known target in a reasonable amount of time. Furthermore, most *in vitro* bioassays are not relevant to *in vivo* or

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clinical conditions regarding either the activity or bioavailability of the compound under examination, not to mention that a metabolite with a specific activity in the pure state may show a different activity when present within a mixture

Innovative strategies are therefore needed to significantly reduce the timeline of bioactive natural product discovery.

All evidence suggests that the most promising workflows for the chemical profiling of natural products should involve, at least in part, a dereplication procedure. Historically, the first definition of the term "**dereplication**" was given by Beutler *et al.* in 1990 as "*a process of quickly identifying known chemotypes*" (Beutler et al. 1990). Their goal was to evaluate the activity of a range of terrestrial and marine plant extracts by using a simple phorbol dibutyrate (PDBu) receptor binding assay and to rapidly identify compounds responsible for this activity without investing time in traditional bioassay-guided fractionation or full structure elucidation procedures.

But what exactly lies behind the term "dereplication" more than twenty years later? Is this definition still relevant today within the context of natural product research? What does a dereplication process involve in terms of methods and techniques? For what purpose is such a strategy really useful? On which natural ressources is it currently applied? How much progress has been made in dereplication workflows over the last years?

In the present review, we would like to address these points by presenting a state-of-the-art report on the various dereplication approaches that have been used until now for the chemical profiling of natural products. A literature survey, covering the period 1990-2014, for the topic "dereplication" or "de-replication" in the Web of Science database revealed 358 hits. Upon closer examination, it emerged that the research areas covered by the matching studies not only include pharmacology/pharmacy, chemistry, or plant sciences, but also molecular biology, biotechnology, microbiology, and food science technology. Another important fact is the huge

increase of published items every year, particularly since 2012, as well as the exponential growth of annual citations (Fig. 1).
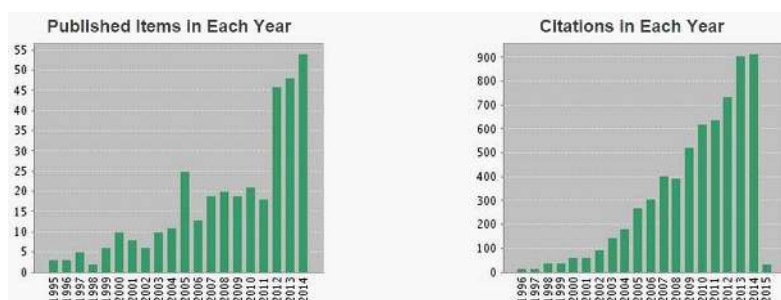

Fig. 1. Citation report obtained with the topic "dereplication" or "de-replication" from Thomson Reuters Web of Science. The latest 20 years up to December 2014 are displayed. (c) Thomson Reuters.

The literature survey was rigorously scrutinized in order to assess the function and usefulness of modern dereplication strategies for bioactive natural product discovery. Of course, the goal of this review is not to impose a definition or a specific way to perform natural product dereplication, but to highlight as objectively as possible that, depending on the natural resources under examination and on the final objectives, dereplication does not correspond to a single, clearly defined step-by-step procedure. In the 358 references found, 14 not dealing with natural products and 30 meeting abstracts were not taken into account. After a brief outline of the most common separation and analytical tools used for natural product dereplication, the critical role of natural metabolite databases and microbial strain libraries will be discussed. The studies covered by the literature survey have been organized in five distinct dereplication categories (annotated from DEREP1 to DEREP5), all involved in the search for novel bioactive substances of natural origin, but each following a particular workflow based on different starting materials, different separation and/or analytical procedures and different structural elucidation approaches.

A comparison chart summarizing these five categories (Table 1) shows that natural product dereplication can be performed as an untargeted workflow for the rapid identification of the major compounds whatever their chemical class in a single natural sample (DEREP1). Frequently, this first strategy is also directly combined with biological assays to accelerate bioactivity-guided fractionation procedures (DEREP2). In other cases dereplication is fully embedded in untargeted metabolomic studies for the chemical profiling of natural extract collections (DEREP3) or for the targeted identification of predetermined compounds (desired or undesired) in natural samples (DEREP4). Finally a distinct dereplication category mainly based on gene-sequence analyses is increasingly used for the taxonomic identification of microbial strains (DEREP5).

Table 1. Summary of the particular features distinguishing from DEREP1 to DEREP5 dereplication workflows.

	DEREP 1	DEREP 2	DEREP 3	DEREP 4	DEREP 5
Goal	Identification of the major compounds in a single extract	Acceleration of activity-guided fractionation	Chemical profiling of crude extract collections	Chemical profiling of target compounds	Taxonomoic identification of microbial strains
Targeted chemical class?	No			Yes	No
Biological assays ?	Independent	Yes, systematically	Independent		
Samples	Single extract		Extract collection	Single extract or collection	Extract collection
Fractionation step ?	In most cases Yes	Yes	No (direct sample analysis)	In most cases No	No (direct sample analysis)
Analytical tools	LC/MS, GC/MS and/or NMR			Mostly LC/MS	Gene-sequencing

Computer tool and statistics for data treatments	May include	Yes, systematically	May include	Yes, systematically
Identification	Metabolite database			Gene database

Technical considerations

As dereplication refers to the rapid identification of known secondary metabolites, it is obvious that reliable, robust, rapid and sensitive analytical methods are required to perform an efficient dereplication procedure. With the remarkable developments of separation sciences, spectroscopic techniques, and high-throughput analytical technologies, valuable resources now exist to structurally characterize natural products, not only as pure compounds, but also within complex mixtures such as crude plant extracts or microbial culture media. This review will start by presenting the most common analytical tools used for the dereplication of natural products. Since many detailed reviews are available for each technique, we will only briefly describe their strengths and weaknesses and focus on their benefits when applied to natural product dereplication.

Analytical chromatographic systems and hyphenation possibilities

High performance liquid chromatography (HPLC) remains by far the most commonly used technique to separate natural compounds at the analytical scale. HPLC is robust, easy-to-use, and applicable to a wide diversity of compounds, even starting from materials of complex chemical composition. More recently, ultra-high pressure liquid chromatography (UHPLC) has enabled a remarkable decrease of separation times while improving sensitivity, resolution, and reproducibility as compared to conventional HPLC. Both techniques, referred to as "LC" in the present paper, are currently applied to natural product analysis for various purposes including quality control, chemical characterization of crude plant extracts or high throughput

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fingerprinting (Wolfender et al. 2010, Eugster et al. 2011). Thin layer chromatography (TLC or HPTLC) is another chromatographic technique frequently used for the preliminary analysis of natural extracts or for the rapid identification of known compounds within simplified mixtures obtained after the prefractionation of a crude natural sample (Hook et al. 1997, Hook 1998, Tan et al. 2011). TLC-based bioautographic methods also enable the rapid localization of biologically active metabolites directly on TLC plates (Hostettmann et al. 2005, Adhami et al. 2013) High speed counter current chromatography (HSCCC), centrifugal partition chromatography (CPC), supercritical fluid chromatography (SFC) or capillary electrophoresis (CE) are less commonly used analytical-scale separation system, and therefore will not be detailed here.

Among post-chromatographic hyphenation possibilities, simple ultraviolet (UV) or photodiode array (DAD) detectors remain limited to the interpretation of peak retention times or UV spectral fingerprints, but can nevertheless be employed in dereplication strategies for both identification and quantification purposes by using standard molecules as references (Wolfender 2009). With the help of chemometric tools, LC/UV-DAD data can be processed by chromatographic band deconvolution, resulting in a significant improvement of chromatographic resolution. This method was recently used to identify several C-glycosylflavone isomers in the traditional herbal medicine *Jatropha gossypifolia* (Pilon et al. 2013). However, due to the very limited structural information recovered from simple UV or DAD detection systems, other more powerful instruments are generally required to assess the structural diversity of natural products. Among them, mass spectrometry (MS) and nuclear magnetic resonance (NMR) described in the next section remain by far the favourites.

MS and NMR-based detection in natural product dereplication workflows

Mass spectrometry is a sensitive, rapid and accurate high throughput detection technique frequently used for the dereplication of natural products. The characterization of natural metabolites is established on the basis of exact mass, elemental composition, adducts and fragmentation patterns. Due to their high sensitivity, MS-based methods also enable the rapid detection of trace-level compounds, meaning that only small amounts of materials is required for successful analyses. LC coupled to high-resolution mass spectrometers such as Time-of-Flight (TOF), Fourier Transform (FT) or Orbitrap devices currently constitute the most powerful "high-throughpout screening" platforms for the on-line identification of metabolites in natural resources (Strege 1999, Wolfender et al. 2000, Shin and van Breemen 2001, Harrigan and Goetz 2005, Bitzer et al. 2007, Moss et al. 2007, Sashidhara and Rosaiah 2007, Nielsen, Mansson et al. 2011, Sarker 2012, Smyth et al. 2012, Carter 2014). Regarding hydrophobic or volatile small molecules, GC/MS is a more appropriate coupling, as demonstrated for instance for the dereplication of fatty acids (Stavri et al. 2004) or flavor and aroma constituents (Molyneux and Schieberle 2007).

Although MS is recognized as a key technology for the identification of natural products, some pitfalls still exist and these problems have to be solved to make MS-based dereplication methods more efficient (Cech and Yu 2013). The major drawback arises from the important variabilities in the raw datasets obtained from one mass analyzer to another, which strongly hampers the creation of exchangeable MS/MS databases. The ionization processes taking place at the input of MS systems to desolvate and charge the analytes are also particularly critical because they vary with the type of ion source (electrospray or atmospheric pressure chemical ionization, for example), and may also vary through ionization suppression or enhancement under matrix effects. A high heterogeneity has been reported after the construction of a Collision-Induced-Dissociation MS/MS spectral library of ubiquitous flavonoids using either hybrid quadrupole time of flight (Q-TOF) or Ion Trap (IT) under various CID energy conditions

(Wolfender et al. 2000). Perfect control of the parameters used to generate informative MS/MS spectra is thus needed for an efficient use of MS detection in the dereplication of natural products.

The interpretation of MS data is another critical issue that limits the speed of dereplication strategies. Efforts are currently underway to develop the computerized treatment of mass spectral data. Software dedicated to the pre-processing of LC-MS data are now available and very useful for peak picking, ion extraction, organization and classification of data. They include for instance Bruker1 Data Analysis and Bruker1 Profile Analysis for Bruker, MassHunter for Agilent, MarkerLynx for Waters or publicly available software such as XCMS, MZmine, and METIDEA which can handle data from different instruments. Among the computer tools that assist metabolite identification, four data mining software are for instance described in a recent review (Hufsky 2014): MetFusion which uses substructures to assess spectral and chemical similarities, ISIS which is based on the prediction of fragmentation patterns for spectra comparison, FingerID which compares molecular structures after prediction of structural features, and FT-BLAST which is based on the use of a fragmentation tree database.

Molecular networking is also a promising computer-based approach to visualize and organize tandem mass spectrometry datasets and to automate database search for metabolite identification within complex mixtures (Garg et al. 2015). Such a tool relies on the observation that structurally similar metabolites share similar MS/MS fragmentation patterns. After collection of MS/MS spectra from a natural sample, the method consists in the construction of a molecular network by measuring the correlations within the produced dataset and establishing a Cytoscape visual representation of the chemical similarities between metabolites. Because structurally similar metabolites share similar fragmentation patterns, molecular families tend to cluster together within a network (Nguyen, Wu et al. 2013, Yang, Sanchez et al. 2013). The

chemical investigation of natural resources by molecular networking has already proven to be a very useful complement to current MS-based dereplication strategies, for instance to highlight the chemical diversity among marine cyanobacteria (Winnikoff, Glukhov et al. 2014), to automatically detect structurally related nonribosomal peptides in filamentous fungi (Klitgaard, Nielsen et al. 2015), or to investigate interkingdom molecular interactions and metabolic exchange processes within microbial communities (Moree, Phelan et al. 2012).

Another promising data mining workflow based on MS^2 precursor lists and targeting only signals related to bacterial metabolism was recently developed to characterize myxobacterial secondary metabolites (Hoffmann et al. 2014). Even direct MS infusion can be used for natural product dereplication if assisted by computer tools. Such an approach was applied to the chemotyping of filamentous fungi strains from culture collections, and to subsequently classify unknown samples, thus avoiding redundancies in the selection of samples to be further investigated (Larsen 2005).

Nuclear Magnetic Resonance (NMR) is the other predominant detection technique used for the dereplication of natural products. It remains by far the most efficient to unambiguously elucidate complex structures of individual small molecules. With the advent of high field magnets, capillary and cryogenic probes, the lower sensitivity of NMR as compared to MS-based analytical methods has been progressively counterbalanced, and even the structure elucidation of minor compounds within mixtures becomes possible (Hu et al. 2008). In addition, sophisticated hardware and software products have been introduced in recent years to promote high-throughput NMR analyses, such as NMRbot Python scripts (Clos et al. 2013). As described in a recent review, these technical improvements along with hyphenation possibilities and their combination with computational treatments is progressively bringing NMR to the front of the powerful resources used in natural product dereplication strategies (Halabalaki et al. 2014).

It should nevertheless be emphasized that solvent and pH effects induce significant chemical shift variations across samples and are important drawbacks of NMR-based methods. These effects can strongly influence the efficiency of natural product dereplication procedures, as demonstrated for instance in a study investigating the molecular structures of caffeoyl quinic acid derivatives (Pauli et al. 1999). The limited spectral dispersion and the complexity of signal patterns, particularly in ^{1}H NMR spectra are also a major NMR challenge. In this sense it was pointed out in a recent paper that unambiguous ^{1}H NMR analyses for dereplication purposes require adequate precision when reporting chemical shifts or ^{1}H-^{1}H coupling constants from ^{1}H spectra, what is not so that easy when working with spectra of natural product mixtures (Pauli 2014). Diffusion ordered spectroscopy (DOSY) was proposed as an efficient alternative to simplify interpretation of NMR spectra of natural mixtures (Williamson et al. 2000, Stessman et al. 2002). It has been shown for instance that 1D-DOSY when combined with 1D or 2D NMR experiments enables the acquisition of full spectral data of high molecular weight polysaccharides directly from crude hot water extracts of mushroom species without the need of any purification step (Politi et al. 2006) Although natural organic compounds also contain carbon atoms, the use of ^{13}C NMR for the analysis of natural extracts remains largely underutilised, mainly because the low abundance of the ^{13}C isotope (1 %) and its low gyromagnetic ratio (25 % of that of ^{1}H) considerably reduce its detection sensitivity. Yet ^{13}C NMR presents several strong advantages for the analysis of natural mixtures: carbon atoms constitute a significant part of all organic molecules, and each ^{13}C position in a molecular structure corresponds to a single resonance on a broadband ^{1}H decoupled ^{13}C NMR spectrum. In addition, the ^{13}C NMR spectral width is significantly higher than that of ^{1}H (220 ppm for ^{13}C and 12 ppm for ^{1}H), what also significantly reduces the occurrence of signal overlaps. Here again with the technical improvements of NMR spectrometers and the emergence of promising methodologies such as dynamic nuclear polarisation, it becomes possible to acquire ^{13}C spectra

of metabolite mixtures with high resolution, good sensitivity, and in a reasonable time. In fact, an increasing amount of work using ^{13}C NMR for the analysis of natural products is emerging (Clendinen et al. 2014, Hubert et al. 2014, Yona et al. 2015).

Particular attention must also be paid to LC/NMR or LC/SPE/NMR methods, which combine the separation performance of liquid chromatography, the concentration effect of SPE, and the structural information provided by NMR. In the case of LC/SPE/NMR, the main advantage is that peak trapping on SPE cartridges significantly increases analyte concentration and thus provides access to 2D NMR data in a short time. The use of LC/NMR has rapidly expanded over the last years for the dereplication of natural products, especially for the direct comparison of metabolite profiles in small extracts using ^{1}H NMR before further investing in more laborious preparative-scale isolation processes. Such strategy greatly helps to avoid re-isolation of already known extract constituents. Several reviews published since the mid 1990s have detailed LC/NMR technical developments and their applications in the field of natural product research (Bobzin et al. 2000, Bobzin et al. 2000, Wolfender et al. 2001, Wolfender et al. 2003, Jaroszewski 2005, Jaroszewski 2005, Wolfender et al. 2005, Wolfender et al. 2006, Queiroz 2009, Brkljaca and Urban 2011, van der Hooft et al. 2013).

The crucial role of natural product databases and computer tools

The success of a dereplication procedure to achieve the rapid characterization of known compounds greatly depends on the availability and quality of natural product databases or strain libraries. Many commercial or public databases of low molecular weight metabolites have been developed over the last 20 years (Corley and Durley 1994, Lopez-Perez et al. 2007, Blunt and Munro 2013, Valli et al. 2013) The more comprehensive databases include the *Dictionary of Natural Products* (DNP) containing over 260,000 molecular structures, the *Dictionary of Marine Natural Products* containing around 48,000 molecular structures drawn from marine

organisms, the *ACD/Labs NMR* and *ACD/Spectrus* databases providing structural and spectral data of many thousands of synthetic and natural compounds, the *UCSD Natural Products Database* containing a large repository of microorganisms and exosymbionts, the *Human Metabolome Database*, *Napralert*, the *NuBBE* database dedicated to the Brasilian biodiversity, the UniProtKB/Swiss-Prot protein sequence database, *KnapSack* which is a comprehensive plant species-metabolite relationship database, and *MarinLit*, *AntiMarin*, *AntiBase* or *Seaweed Metabolite* databases dedicated to marine and/or microbial molecular structures. This is not to mention all the locally-built libraries fragmented and scattered in many laboratories worldwide. There is a crucial need today to expand and standardize these natural products databases.

A number of studies found in the literature survey were performed with the sole objective of database construction and implementation. Many of them were based on molecular structures, while others were focused on MS fragmentation patterns, bioactivity or taxonomic data (Fredenhagen et al. 2005, Konishi et al. 2007, Liu et al. 2008, Overy et al. 2008, Rojas-Cherto et al. 2012).

In addition to databases, computer tools and multivariate statistics have considerably improved the automation of analytical data processing and have substantially contributed to the current enhancement of natural product dereplication strategies (Hansen et al. 2005, Wolf and Siems 2007, Jarussophon et al. 2009, Ng, Bandeira et al. 2009, Khosrokhavar et al. 2010, Wolfender et al. 2010, Chlipala et al. 2011, Eugster et al. 2014, Kokkotou et al. 2014). Strong advances in the area of Computer-Assisted Structure Elucidation (CASE) systems, which combine artificial intelligence software with spectroscopic data to automatically generate molecular structures, have also dramatically accelerated structure elucidation procedures and have improved the reliability of dereplication results (Jaspars 1999, Steinbeck 2004, Elyashberg et al. 2009).

Finally, spectral pattern recognition strategies based on the organization of spectral data according to chemical similarities are also powerful complements to natural product

dereplication workflows. In this case spectroscopic data of metabolite mixtures are required along with spectra obtained from known standards, well-characterized organisms or prediction tools, preferably organized into databases. The utility of pattern recognition tools for natural product dereplication was for instance demonstrated for the chemical characterization of a diverse array of marine and terrestrial microbial samples using a MS-based method (Yang et al. 2013), or for the chemical characterization of plant extracts using ^{13}C NMR combined to hierarchical clustering analysis (Hubert et al. 2014).

Dereplication and metabolomics: Where to draw the line?

Before entering the literature survey, some thought should be given to the term "chemical profiling" as well as to the obvious connection between dereplication and "metabolomics". Of course each of these two terms alone deserves detailed discussion, as their use can be ambigous depending on the context. For the sake of clarity, we would like to define the meaning of "chemical profiling" within the context of the present review, and also to highlight that a range of metabolomic studies are fully associated with natural metabolite dereplication workflows.

The term "chemical profiling" will be defined here in a very broad sense as the systematic detection and/or identification and/or quantification of a range of natural compounds. Depending on the final purpose and on the analytical technique available, chemical profiling can be performed as a fingerprint analysis or as a targeted profile. A fingerprint analysis represents a global analysis where all detected metabolites are not necessarily identified. In a targeted chemical profiling approach, a predefined number of compounds or a particular chemical class of compounds is investigated and the molecular structures are identified.

Metabolomics, which is defined as the qualitative and quantitative analysis of the whole set of low molecular weight metabolites present in a biological system (Fiehn 2002, Zhang et al. 2012), also falls within the scope of a chemical profiling. However, more specifically,

metabolomics is an interdisciplinary field that combines high resolution analytical systems, multivariate statistics and data mining tools, chemical and biological knowledge, and sometimes metabolic network modeling in an attempt to understand metabolic pathways, gene-function relationships, or states of an organism in response to environmental changes, drug perturbations, phylogeny, genotypic or phenotypic variabilities. Metabolomics workflows are strongly context-dependent and have been used over the last years in so many different fields that a range of experimental design methodologies have been developed with specific lines regarding sample preparation, data analysis or metabolite identification strategies. To keep it simple, we will only distinguish "targeted" from "untargeted" metabolomics studies. Typically, a targeted metabolomic approach is used to analyze a set of defined metabolites in a sample, while a much more comprehensive "untargeted" metabolomics approach consists of the detection and/or quantification of as many metabolites as possible without *a priori* knowledge of metabolite targets. In this latter case, samples and metabolites are generally classified according to spectral patterns. In the field of natural product research, both targeted and untargeted metabolomic approaches have already demonstrated strong potentialities, mainly for the quality control of herbal medicines, for the determination of chemical markers in plant species, or to assess the correlations between bioactivity and composition (Fiehn 2002, Yuliana et al. 2011, Sheridan et al. 2012, Zhang et al. 2012, Cox et al. 2014, Seiber et al. 2014). Of course the use of metabolite databases for the identification of the metabolites was a key aspect of all of these studies, and although not commented on explicitly, a dereplication process was in fact involved in the metabolomics study every time that a known metabolite was identified with the help of a database. It must be mentioned here that in dereplication, metabolite identification is often only a putative identification. Several articles have suggested ways to evaluate the level of confidence in metabolite identification (Dunn, Erban et al. 2013). For instance the Chemical Analysis Working Group of the Metabolomics Standards Initiative

(http://msi-workgroups.sourceforge.net) has defined four different confidence levels, with level 1 corresponding to confidently identified compounds by comparing multiple physicochemical properties of a pure standard to those of the metabolite of interest, level 2 corresponding to putatively annotated compounds, level 3 corresponding to putatively annotated compound classes and level 4 corresponding to unknown compounds (Sumner, Amberg et al. 2007, Creek, Dunn et al. 2014). Implementation of such levels, mainly in the field of metabolomics, would also be useful for the natural product research community.

Dereplication of natural products: one concept involved in different strategies

Rapid identification of the major compounds in a single natural extract regardless of chemical class (DEREP1)

A very common way to perform dereplication of natural products today is to establish the chemical profile of a single natural resource using a hyphenated analytical system and to directly compare the obtained spectroscopic data with libraries of natural metabolites. This dereplication approach will be annotated "DEREP1" in the present review and is illustrated in Fig. 2.
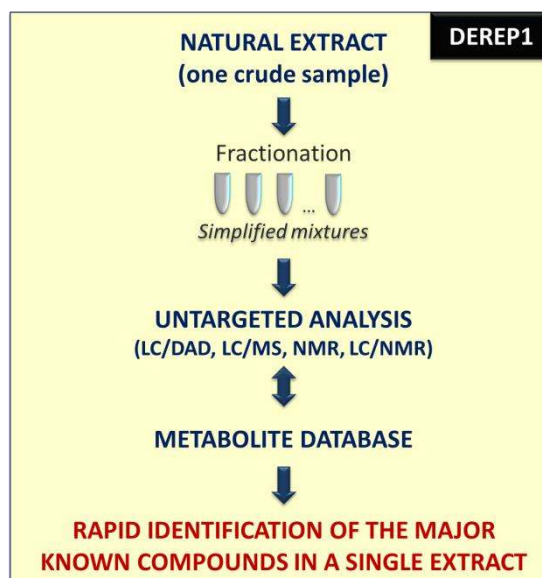
Fig. 2. DEREP1: an untargeted dereplication workflow aiming at the rapid identification of the major known compounds whatever their chemical class in a single natural sample

The starting natural resource is most often fractionated before the analytical step and the dereplication procedure can be achieved independently from bioassay experiments. In this way, a global view of the main chemical classes present in a natural species can be easily obtained and theoretically the major known compounds rapidly identified. Among the published works mentioning the term "dereplication" between 1990 and 2014, 50 studies summarized in Table 2 were based on a DEREP1 workflow (16 % of the total examined articles). More than two-thirds of these studies were carried out on plant extracts, while a minority delt with marine sponges, brown algae and cyanobacteria extracts. This distribution is logical considering that databases containing spectroscopic data of plant-derived metabolites are substantially more developed than those related to less explored marine or microbial species. The analytical tools involved in these studies were mainly based on high resolution LC/MS or NMR, and molecular structure assignments were usually performed by directly entering spectral data (exact mass or molecular formula in MS, chemical shifts in NMR, UV spectra or log P-based estimation of the chromatographic retention time in LC/DAD) into a database for automatic search. In several studies the strength of the procedure was reinforced by combining the analytical step with

computational treatments in order to detect spectral patterns before searching molecular structures into metabolite databases (Boudreau et al. 2012, Hubert et al. 2014).

It can also be noted from Table 2 that, even if flavonoids and phenolic acids remain by far the most investigated compounds, a diversity of other chemical classes including anthrones, iridoids, terpenoids or fatty acids have also been successfully identified in these DEREP1 studies. Another advantage is that although dereplication does not provide original information on novel chemical structures, previously known compounds can still be identified in genus or species in which they are not *a priori* expected. For instance, flavonoid glycosides already known in a range of plant species were identified for the first time in the genus *Lasiopetolum* by using a LC/NMR-based DEREP1 procedure (Timmers and Urban 2011). Of course, the efficiency of such dereplication approach strongly depends on the quality of spectral libraries. As mentioned above, significant efforts are currently being to develop and especially to standardize comprehensive databases containing structures and spectroscopic data (MS or NMR) of natural products.

Table 2. Dereplication strategies published from 1990 to 2014 using a DEREP1 workflow (from oldest to most recent).

Identified chemical classes and natural sources	Analytical technique	Reference
Representative set of 46 plant and microbe-derived metabolites	ESI-MS, API-MS	Zhou and Hamburger 1996
An alkaloid and a sesquiterpene previously purified	NMR, computer-assisted identification using the DNP	Bradshaw et al. 2001
Three pentaketides and four fungal pigments from a deep water marine-derived fungal culture	1D and 2D NMR	Gautschi et al. 2004
Ent-labdane diterpene glycosides from polar extracts of *Potamogeton* species	LC/MS, LC/MS^2, LC/UV, LC/NMR	Waridel et al. 2004
Enantiomeric 1-acetoxychavicol acetates and carvones	Chiral Lanthanide shift reagents in NMR	Jaki et al. 2004
Flavonol glycosides and cardenolides from flower, leaf, root, and stem extracts of *Kanahia laniflora*	LC/DAD/SPE/NMR	Clarkson et al. 2005

Aspirochlorine and two new antifungal derivatives from *Aspergillus flavus*	LC/UV/ELSD/MS, NMR	Klausmeyer et al. 2005
Cyclic peptides from a crude fungal extract	LC/UV, HSCCC	Dalsgaard et al. 2005
Senkyunolide A, butylphthalide, neocnidilide, Z-ligustilide and several phthalide dimers from the TCM *Ligusticum chuanxiong*	LC/UV, LC/MS, LC/NMR	Zschocke et al. 2005
Ursene triterpenes from *Diospyros dendo* with antibacterial activity against *Pseudomonas aeruginosa*	Capillary-scale NMR	Hu et al. 2006
Panel of 179 natural standard molecules	^{1}H and ^{13}C NMR	Dunkel and Wu 2007
Huperzine A and a *Lycopodium* anti-Alzheimer drug lead alkaloid	^{1}H NMR, spectral prediction	Niemitz et al. 2007
Naphtodianthrones, phloroglucinols, flavonoids and phenolic acids from Greek *Hypericum perforatum*	LC/DAD/SPE/NMR, LC/UV/ESI-MS	Tatsis et al. 2007
Several bioactive compound from the Chinese herbal formula Qi-Xue-Bing-Zhi	LC/DAD/MS, computer-assisted data processing (WiseProcessor) and library	Liu et al. 2008
Flavonoids, sesquiterpene lactones and phenolic acids from Brasilian *Lychnophora ericoides* leaves	LC/DAD/MS and MS^2	Gobbo-Neto and Lopes 2008
Pentaprenylated *p*-quinol from an antitumour extract of the marine sponge *Dactylospongia* sp.	LC/MS, LC/NMR	Dias and Urban 2009
Bastadins, 19-Hydroxyaraplysillin-I-N^{20}-Sulfamate and Araplysillin-I-N^{20}-sulfamate from the marine sponge *Lanthella flabelliformis*	FTICR/MS, MS^n	Motti et al. 2009
Oxy-polyhalogenated diphenyl ethers from *Dysidea* sponges	NMR, X-ray crystal	Calcul al. 2009
Potent antiplasmodial quinolinone alkaloids from *Haplophyllum acutifolium*	LC/DAD/MS/SPE/NMR	Staerk et al. 2009
Fatty acids from the antifouling marine sponge *Cholla delitrix*	TLC, GC/MS, NMR	Castellanos et al. 2010
Chlorinated cylindrocyclophanes from a Nostoc cyanobacteria collected in a parkway of Chicago with antiproliferative activity	LC/UV/MS	Chlipala et al. 2010
Five cyclic tetrapyrrolic photosensitisers from *Phaeanthus ophtalmicus* leaves	TLC, LC	Tan et al. 2011
Spiro compounds and tracheloside from *Carthamus oxyacantha*	HPLC/DAD/MS/SPE/NMR	Johansen et al. 2011
719 microbial natural product and mycotoxin standards	LC/DAD/TOF-MS, MS/MS under different ion-source settings	Nielsen et al. 2011
Linear polyketides and tubercidin from *Actinopolyspora erythraea*	1D and 2D NMR	Zhao et al. 2011
Quantification of structurally diverse standard natural products	ELSD	Adnani et al. 2012

Tiliroside derivatives (on-line) and flavanoid glycosides (off-line) from *Lasiopetolum macrophyllum*	On-line LC/NMR, off-line LC, NMR, MS	Timmers and Urban 2011, Timmers and Urban 2012
Alkaloids, amides or esters of hydroxycinnamic acid and betain from *Cimicifuga racemosa* (black cohosh)	LC/MS	Nikolic et al. 2012
Phenolic allopyranosides, amide allopyranosides, and phenolic compounds from the rhizomes of *Cimicifuga heracleifolia*	LC/NMR, LC/MS	Yim et al. 2012
Stem-specific metabolites including three stilbenoids from *Vanda coerulea* (Orchidaceae)	HPTLC/MS	Cakova et al. 2012
Beilschmiedic acid derivatives from the leaves of a Gabonese *Beilschmiedia* species	Micro-CryoProbe NMR	Williams et al. 2012
Hectochlorin derivatives and several jamacamides from the cyanobacteria *Moorea producens*	MS^2	Boudreau et al. 2012
Iridoid and triterpenoid glycosides from *Premna fulva* leaves and stems	LC/DAD/MS	Niu et al. 2013
Phenolic glycosides, dimeric phenylpropanoid glucoside, saponins, and fatty acids from *Phyteuma orbiculare* leaves	LC/DAD/MS, NMR	Abbet et al. 2013
Two known naphthocoumarins and one new naphthocoumarin from *Streptomyces sporoverrucosus*	HPLC-PDA/LC-MS combined to DNP, 2D NMR	Jain et al. 2013
Resorcinol and two polyene derivatives from the brown algae *Cystophora tondosa*	LC/NMR	Urban and Timmers 2013
Spiro compounds from *Carthamus oxyacantha* (wild safflower) and griseofulvin and analogues from the endophytic fungus *Penicillum namyslowski*	LC/NMR	Johansen et al. 2013
Leporizines from an *Aspergillus* sp. strain	NMR	Reategui et al. 2013
Phenolic glycosides, monoterpene lactone, spermine derivatives and fatty acids from *Cirsium spinosissimum*	LC/DAD/MS, microprobe NMR	Abbet et al. 2014
Isomeric butanoates and pentanoates of long-chain 1-alkanols from the essential oil of *Scandix pecten-veneris*	GC/MS	Radulovic et al. 2014
Triterpenes, saponins, phenolic acids, flavonoids and ellagic acid derivatives from the bark of *Anogeissus leiocarpus*	^{13}C NMR, HCA	Hubert et al. 2014
Polyketides, non-ribosomal peptides, terpenes, meroterpenoids and four novel isomers of the known anticancer compound asperphenamate, from marine-derived strains of *Aspergillus*, *Penicillium*, and *Emericellopsis*	LC/DAD/QToF-MS, MS/MS	Kildgaard et al. 2014
Hydroxylated fatty acids, antimycin compounds and three butenolides from a sponge-derived *Streptomyces* sp. with antibacterial and antifungal activities	LC/MS	Viegelmann et al. 2014
Indole and naphthoquinone derivatives from a novel myxobacterial strain isolated from compost in Germany	MS, NMR	Jansen et al. 2014
Diplosporin, coriloxin, mellein and agistatine derivatives from a *Xylaria* sp. isolated as an endophyte from a surface-sterilized Concord grape leaf (*Vitis labrusca*)	LC/MS	Ibrahim et al. 2014

Dioxomorpholine, okaramine, and aflavinine derivatives and three novel structures of mixed biosynthetic origin named aculenes A-C from the black filamentous fungi *Aspergillus aculeatus*	LC/DAD/MS, NMR	Petersen et al. 2014
Four macrotetrolides homologous to nonactin and three related linear dimers from *Streptomyces* sp.	MS, LC/MS[2]	Crevelin et al. 2014
Six major depsides from the lichen *Pseudevernia furfuracea*	^{13}C NMR, HCA	Oettl et al. 2014
Ten known compounds, including angucycline, diketopiperazine and β-carboline and three novel derivatives produced in the culture medium of *Actinokineospora* sp. EG49 grown in co-culture with *Nocardiopsis* sp.	NMR	Dashti et al. 2014

Dereplication workflows involved in bioactivity-guided fractionation procedures (DEREP2)

A widely used strategy in the search for new bioactive molecules among natural products consists of the so-called bioactivity-guided fractionation procedures, in which pharmacological or biological assays are performed to target the isolation of active constituents. They were initially developed to avoid wasting time and resources in considering inactive, thus arbitrarily implying "uninteresting" compounds, and focusing only on the fractions or metabolites with a predefined biological activity (Agarwal 2014). Once fractions obtained from a crude extract have been revealed active by biological assays, classical separation and analytical techniques are applied to isolate and identify the individual active substances (Duarte et al. 2012, Smyth et al. 2012). Such procedures are currently very common in natural product research, and a range of chemical, enzymatic, and *in vitro* biological tests (antioxidant, antimicrobial, antifungal, anti-inflammatory…) are routinely available in many laboratories (Hostettmann et al. 2001, Cos 2006).

However, the identification of new compounds by bioactivity-guided fractionation procedures requires a multi-step workflow, and often a great deal of work is done to purify the bioactive fractions and finally discover previously known compounds (Agarwal et al. 2014).

Consequently, dereplication also constitutes a key approach to overcome this frequent issue of known compound rediscovery in bioactivity-guided fractionation procedures.

In the literature survey, 52 studies (17 % of the total examined articles) were dedicated to the identification of natural metabolites on the basis of bioactivity-guided fractionation procedures. These approaches will be annotated DEREP2 in the present paper. It is obvious that DEREP1 and DEREP2 workflows are not that different, except that DEREP2 ones are systematically focused on active natural samples as determined by biological assays. These studies are summarized in Table 3 and their common global workflow is illustrated in Fig. 3.
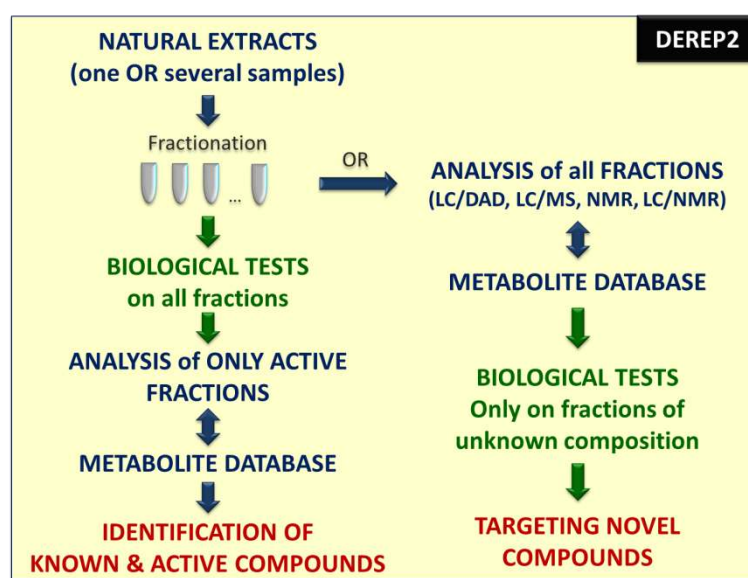
Fig. 3. DEREP2: A dereplication workflow for the acceleration of bioactivity-guided fractionation procedures

Among these studies, a vast majority was again focused on plant extracts and a minority on marine and microbial extracts. In some cases, dereplication was performed at an early stage in an attempt to rapidly distinguish known constituents from those presenting novel or unusual spectroscopic features before undergoing multi-step isolation procedures. From an analytical point of view, DEREP2 workflows are most often based on LC/MS^n and/or NMR to analyze biologically active fractions obtained after the pre-fractionation of the crude extract. As

described in Table 3, the resulting molecular masses, fragmentation patterns or NMR chemical shifts are then compared to data from the literature or submitted to commercially available or in-house natural product databases for the identification of known compounds. Analytical data that does not correlate with any known compound thus theoretically corresponds to novel molecules that can be further investigated and ultimately find application as new active substance. For example, this strategy was thoroughly described in a study investigating antibacterial substances in the leaves of *Melicope vitiflora* by using an *in vitro* biological assay in combination with a MS-based dereplication method (O'Donnell et al. 2009). More indirectly, dereplication was also reported useful when combined with bioactivity-guided fractionation procedures to rapidly set aside undesired constituents. For instance, the dereplication of sulphated polysaccharides was achieved in two studies investigating new potent anti-HIV compounds, because many of them were already extensively described in the literature as having an anti-HIV activity and thus were not considered as interesting compounds (Beutler et al. 1993, Harnett et al. 2005). In this way, the tedious isolation of undesired constituents could be avoided, so that isolation, structure determination, and pharmacological investigations could be carried out only on bioactive and novel compounds.

It must be pointed out that most bioactivity-guided fractionation procedures are based on the theory that the activity shown by a mixture of metabolites results directly from the sum of activities of individual metabolites. However, it is becoming evident that the biological effects of a natural extract most likely result from a multi-component synergism (Pavana et al. 2009, Wagner 2011, Yuliana et al. 2013). Another limitation reported for bioactivity-guided fractionation is the irrelevance of most *in vitro* bioassays as compared to *in vivo* or clinical conditions regarding the efficacy and bioavailability of the samples under examination (Houghton 2007). Potentially interesting compounds may also be missed just because they are not active on the selected biological assays. Research strategies for the discovery of novel

bioactive molecules among natural products are thus progressively re-evaluated to consider

more than one active constituent within natural resources (Wang et al. 2012).

Table 3. Dereplication strategies published from 1990 to 2014 using a DEREP2 workflow
(from oldest to most recent)

Natural materials	Biological assays	Compounds identified	Analytical technique(s)	Ref
Lyngbya majuscula (filamentous cyanobacteria) and *Croton cuneatus* (plant)	Phorbol dibutyrate (PDBu) receptor binding assay	Debromoaplysiatoxin in *Lyngbya majuscula* and a complex of potent phorbol esters in *Croton cuneatus*	HPLC/UV	Beutler et al. 1990
Marine invertebrates	HIV inhibition test	Sulphated polysaccharides (for removal)	Precipitation method	Beutler et al. 1993
Thevetia ahouia (wood)	Cytotoxicity against a panel of human cancer cell lines	Cardenolide glycosides	UV, IR, MS, NMR	Decosterd et al. 1994
Marine sponge *Agelas axifera*	*In vitro* radioreceptor binding assay on protein kinase C	Agelasines	NMR	deVries et al. 1997
Azadirachta excels (stem bark)	Cytotoxicity against KB cell lines	Meliacin-type limonoids	LC/ESI-MS	Cui et al. 1998
Semecarpus anacardium	Cytotoxicity against a panel of human cancer cell lines	Alkenyl catechols	HPLC/ESI-MS	Shin et al. 1999
Five crude plant extracts	Radioligand Receptor Binding Assays; AChE Inhibitory Activity; *in vitro* anti-asthmatic assay	Galanthamine, theobromine, caffeine, fatty acids, phenolic acids and flavonoids	CPC, TLC	Ingkaninan et al. 2000
Acnistus arborescens (leaves)	Cytotoxicity against a panel of human cancer cell lines	Withanolides	NMR, MS	Minguzzi et al. 2002
Kielmeyera albopunctata (bark)	Cytotoxicity against KB cell lines	Coumarins	LC/MS	Scio et al. 2003
Plant extract spiked with 2 naturally occurring PDE inhibitors	Continuous-flow enzymatic assay	Phosphodiesterase (PDE) inhibitors	LC/fluorescence	Schenk et al. 2003
Artocarpus kemando (stem bark)	Cytotoxicity against KB cell lines and DNA strand-scission activity	Prenylated flavonoids	HPLC/ESI-MS	Seo et al. 2003
Plant extract spiked with tetramisole	Continuous-flow enzymatic assay	Phosphatase inhibitors	LC/MS (on-line)	Schenk et al. 2003
In vitro marine culture of the strain	Antifungal activity against *Candida albicans*	Griseofulvin	ESI-IT/MS and MSn	Petit et al. 2004

Penicillium waksmanii Zaleski				
Humulus lupulus	Antimycobacterial activity against *Mycobacterium fortuitum*	Fatty acids	NMR, GC/MS	Stavri et al. 2004
Macrococculus pomiferus (stems)	Cyclooxygenase-2 inhibition test	Dibenzylbutyrolactone lignans and seven known compounds	NMR, MS	Su et al. 2004
Punica granatum (peel)	Estrogen receptor binding assay	Luteolin, quercetin and kaempferol	LC/MS (on-line)	van Elswijk et al. 2004
Sutherlandia frutescens and *Lobostemon trigonus*	HIV inhibition test	Sulphated polysaccharides (for removal)	Precipitation method	Harnett et al. 2005
Antibacterial lead compounds	Live/Dead bacterial viability kit containing two fluorescent nucleic acid stains	Nine known antibiotics and 14 novel lead compounds	Fluorescence microscopy	Singh 2006
Cleistopholis patens	Antibacterial activity against *Staphylococcus aureus*	Three new and five known acetylated oligorhamnosides	capillary-scale NMR, ESI-MS	Hu et al. 2006
Six *Terminalia* species	*In vitro* antifungal activity (*C. albicans*, *C. neoformans*, *A. fumigatus*, *M. canis* and *S. schenkii*)	Non-polar antifungal compounds	bioautography	Masoko and Eloff 2005
Dracaena angustifolia (leaves)	Anti-mycobacterial test	Three compounds (ergosterol-5,8-endoperoxide, linoleic acid and E-phytol)	TLC	Case et al. 2007
Alangium longiflorum	Inhibition of transcriptional activation pathway in human cancer cell lines	Tubulosine and two derivatives	NMR	Klausmeyer et al. 2008
Ophiorrhiza trichocarpon	Inhibition of transcriptional activation pathway in human cancer cell lines	Camptothecin and three analogues	LC	Klausmeyer et al. 2007
Parthenium hispitum	Inhibitory activity against hepatitis C virus	Five new and four known oxygenated hydroxy-pseudoguaienolides	LC, capillary-scale NMR	Hu et al. 2007
Petiveria alliacea	Cytotoxicity against several tumor cell lines	13 possible known compounds	LC/MALDI-TOF-MS	Uruena et al. 2008
Distephanus angulifolius	*In vitro* antiplasmodial assay	Chlorogenic acid analogs, steroid saponins and sesquiterpene lactones	HPLC/DAD/MS, SPE/NMR	Pedersen et al. 2009
Albany Molecular Research Inc. (AMRI) natural product library	*In vitro* activity against multi-drug resistant *Staphylococcus aureus* and counter screening	Mutactimycin E	NMR	Hopp et al. 2008

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	for cytotoxicity against the human HepG2 cell line			
Melicope vitiflora	Antibacterial activity against methicillin-resistant-*Staphylococcus aureus* and *Micrococcus luteus*	12 known compounds	TLC/ESI-MS^n	O'Donnell et al. 2009
Cimicifuga racemosa	Serotonergic activity using a 5-HT(7) bioassay	Cimicifugic acids	NMR (structure-based spin-pattern analysis)	Goedecke et al. 2009
Myxobacterial extracts	Bacterial bioassay based on a whole-cell bioluminescent reporter gene assay	Inthomycin A	TLC, LC/MS, LC/NMR	Kreiss et al. 2010
Aegle marmelos (Bael tree)	Inhibition of transcriptional activation pathway in a human breast tumor cell lines	Two protolimonoids	NMR	Li et al. 2011
Cacospongia mycofijiensis (marine sponge)	Cytoskeletal profiling, cytotoxicity and antiparasitic activity	Twelve known and four new compounds	LC/MS/UV/ELSD	Johnson et al. 2011
Syzygium polyanthum leaves	Photocytotoxicity	Two new phloroglucinol derivatives and five known pheophorbides	*Data not found*	Har et al. 2012
Medicinal plants	*In vivo* zebrafish bioassays applied on microfractions	Anticonvulsant and antiangiogenic compounds	Microflow NMR, UHPLC/MS	Challal et al. 2012
Diospyros peregrina fruits	1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay	Luteoline-4'-methyl-ether-7-*O*-glucoside and quercetin-3-*O*-(glucosyl)- glucoside	TLC-bioautography	Sahu et al. 2012
Ficus coronata	Antibacterial activity against methicillin-resistant-*Staphylococcus aureus* and *Micrococcus luteus*	Skimmianine, 7-hydroxycoumarin, dihydrocoumarin, bergapten, chalepin, rutamarin, suberenol	HPLC-ESI-MSn and ESI-MSn	Smyth et al. 2012
Marine-derived *Streptomyces* sp. culture	Cytotoxicity	One unknown and one known compounds	microprobe NMR, ESI-MS	Mahyudin et al. 2012
Elaeocarpus chinensis	Cytotoxicity against a human colon cancer cell line	Epoxycucurbitacin derivatives and cucurbitacins	LC/MS	Pan et al. 2012
Seven most active among 289 fungal extracts	*In vitro* bioactivity towards leukemia cells	Ophiobolins	LC/DAD/MS, explorative SPE	Bladt et al. 2012, Bladt et al. 2013

Tanacetum parthenium, *Vinca major*, *Salvia officinalis*, and *Corydalis cava*	*In vitro* membrane permeability assay for the blood brain barrier	Corydaline, Vincamajine, 11,13-dihydroparthenolide, tetrahydropalmatine, majdine, parthenolide, methyl carnosate, epiisorosmanol	NMR, LC/MS	Konczol et al. 2013
Aglaia perviridis	Cytotoxicity against a human colon cancer cell line	Eight new compounds, and 16 known compounds	LC/MS	Pan et al. 2013
Vulcanodinium rugosum (dinoflagellate)	Cytotoxicity test against Neuro2A and KB cell lines	Nakijiquinone A, N-carboxy-methyl-smenospongine and stachybotrin A	MS	Geiger et al. 2013
Carteriospongia sp. and another similar sponge	Antiproliferative activity against three cancer cell lines	Homoscalarane-derived sesterterpenes	UV, IR, NMR	Harinantenai na et al. 2013
Lipophilic extracts from Chinese mangrove endophytes	*In vitro* anti-malaria and cytotoxicity	Polyketides, lipids, diaporthochromones, mycotoxins lipids	LC/MS, NMR	Calcul et al. 2013
Culture extract of *Xylaria* sp., an endophytic fungus from *Ficus pumila*	*In vitro* antimicrobial activity against human and phytopathogenic bacteria and fungi	Benzoic acid derivative	TLC, LC/DAD/MS	Rakshith et al. 2013
Premna odorata leaves	*In vitro* antimycobacterial test	1-heneicosyl formate, β-sitosterol, stigmasterol and diosmetin	GC/MS	Lirio et al. 2014
Three active plant extracts from French Polynesia including *Alphitonia zizyphoides*	*In vitro* anti-fungal activity against *Candida albicans*	Betulinic acid	MS, NMR	Bertrand et al. 2014
Premna odorata Blanco leaves (from Philippines)	*In vitro* antimycobacterial activity	1-heneicosyl formate, p-sitosterol, stigmasterol and diosmetin	GC/MS, NMR	Lirio et al. 2014
Eriodictyon angustifolium and *Thuja occidentalis*	*In vitro* ependymoma cell line bioassay	Flavonoids and diterpenoids	LC/MS/EL SD/DAD database	Yang et al. 2014
Piptocoma antillana leaves and twigs	*In vitro* antiproliferative test against ovarian cancer cells and antiparasitic assay against *Plasmodium falciparum*	Two novel goyazensolide-type and two known sesquiterpene lactones	MS, NMR	Liu et al. 2014
Trigonella foenum-graecum seed extract	*In vitro* SIRT6 (histone deacetylase involved in age-associated metabolic disorders) binding test	Orientin and seventeen other compounds	LC/MS	Singh et al. 2014

Dereplication procedures directly applied to crude extract collections (DEREP3)

The ability to directly identify molecular structures within crude extracts of natural products has become a critical step, either to cope with potent synergistic effects between constituents during biological evaluation, or even just to ensure that the material under examination presents an interest before going further with fractionation or purification steps. Here again, at the earliest stage of natural resource analysis, dereplication strategies have proved their value. It is obvious that the quick identification of known compounds directly in crude extracts is very ambitious because of the highly complex mixtures of non-fractionated samples. However, with the recent analytical advances, mainly in terms of resolution improvement and hyphenation capabilities, a range of efficient analytical systems are available today for the detailed analysis of complex mixtures (Funari et al. 2013, Michel et al. 2013, Carter 2014, Halabalaki et al. 2014). We will note as "DEREP3" all studies (n=50, 16 % of the total examined articles) found in the literature survey in which natural product dereplication consisted of the direct analysis of crude extract collections, either in combination with bioassays or not, but in all cases without fractionation or isolation of individual constituents. These studies are summarized in Table 4 and their workflow is illustrated in Fig. 4.
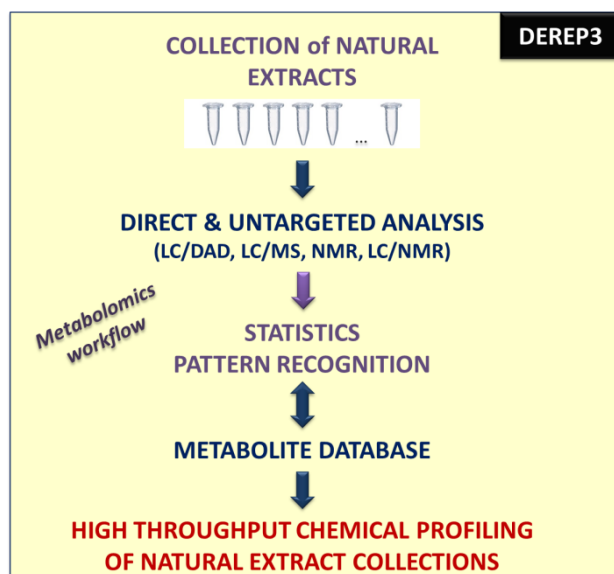
Fig. 4. DEREP3: a dereplication approach fully embedded in untargeted metabolomic analyses of natural extract collections

It emerged from the literature survey that different objectives could be reached by means of DEREP3-based workflows. Many of them aimed at the chemical profiling of crude extract collections by using pattern recognition tools or statistical treatments in an attempt to facilitate the visualization of either known or unusual spectroscopic features. In this way a substantial amount of time can be saved by focusing only on novel substances. It was reported that a hierarchical clustering analysis of GC/MS data obtained from 500 bacterial isolates allowed the selection of samples with a high probability of containing unknown natural products while avoiding the unnecessary analysis of samples of similar composition (Boroczky et al. 2006). In another interesting study, a collection of 30 New Zealand algae extracts were analyzed by a 2D NMR method based on the addition of HSQC spectra to construct a "stacked HSQC digital mask" displaying strong similarities between spectra as intense peaks (Popplewell and Northcote 2009). In order to enhance the unique signals present, implying potentially novel compounds, the mask was substracted from each individual HSQC spectrum. In this way a new bromophenol, colensolide A, was isolated from the red algae *Osmundaria colensoi* together with the known lanosol (2,3-dibromo-4,5-dihydroxybenzyl alcohol) and four of its derivatives.

A range of untargeted metabolomic studies fit perfectly with DEREP3 strategies and provide interesting perspectives especially in the chemical profiling of underexplored marine or microbial extracts. Investigation of marine species and microbes are indeed developing at an exponential rate, mainly based on phylogenetic analyses for drug lead discovery, but the vast array of available species and strains together with their unusual metabolite profiles as compared to plant species make it difficult to evaluate their metabolite composition (Rocha-Martin et al. 2014). It was reported in 2012 that the investigation of filamentous fungi for anticancer drug leads resulted in the isolation and characterization of only 140 compounds over the four previous years, and that only 30% of these represented new chemical entities (El-Elimat et al. 2012). Dereplication of microbial culture collections using DEREP3-based metabolomic procedures can therefore be helpful to classify strain collections *via* a chemotyping approach. Moreover, DEREP3-based strategies should accelerate the evaluation of metabolite diversity, mainly by avoiding redundancy in the identification of metabolites produced by the species under examination and by maximizing microbial natural product libraries that can be generated from collections of microorganisms (Berdy 2005, Larsen et al. 2005, Silver 2006, Liu et al. 2012, Tawfike 2013, Ito and Masubuchi 2014). The direct analysis of fungi directly in fermentation extracts using LC/MS, pattern recognition tools and databases was reported in several papers as an efficient alternative to discover novel molecules with unknown activities (Martin et al. 2012, El-Elimat et al. 2013). In another example, a metabolomic approach combining HR-FTMS and NMR spectral data to PCA, HCA and OPLS-DA statistical treatments was used for the dereplication of metabolites in antitrypanosomally active sponge-associated bacterium *Actinokineospora* sp. obtained from four different fermentation conditions, the main objective being to identify the best culture one-strain-many-compounds condition for the isolation of novel bioactive metabolites (Abdelmohsen 2014).

Other interesting studies performing "co-cultures" of microorganisms to enhance the diversity of metabolite production *via* the activation of silent genes have followed a DEREP3 workflow to rapidly determine the presence of novel compounds while leaving aside those already known (Butler et al. 2012, Bertrand et al. 2013). For example, ten known compounds were identified by dereplication in a crude extract of the sponge-derived actinomycetes *Actinokineospora* sp. while three unexpected natural products not detected in the single culture were identified when *Actinokineospora* sp. was grown in co-culture with *Nocardiopsis* sp. (Dashti, Grkvic et al. 2014).

Another interesting aspect of DEREP3 strategies arises from the possibility to directly correlate biological properties to chromatographic and spectroscopic features of the crude analyzed samples. In this case, the purpose is to link the chemical characteristics of natural compounds to a specific biological activity. With the help of the ChemGPS-natural product database, it was shown that the inhibition of cyclooxygenase enzymes involved in inflammatory processes is frequently correlated with the presence of at least one ring in the metabolite structure. Fragments exhibit structural rigidity, and compounds have a relatively large molecular volume (Larsson et al. 2005). In another example, a screening technology combining on-line biological analysis with the resolution of LC/MS for structure elucidation was developed to directly analyze estrogen-receptor binding compounds in complex mixtures of a large plant extract library (Schobel et al. 2001). LC/MS signals of compounds from 22 phenolic extracts of extra virgin olive oil showing an activity against a breast cancer cell line were also identified by applying a correlation analysis between particular spectral features and the observed activity. From this method a model was estimated to predict the activity of new samples (Roldan et al. 2013). Optimization of such strategies in future research efforts could significantly accelerate the discovery of novel active substances and help our understanding of natural products with respect to structure-activity relationships.

Table 4. Dereplication strategies published from 1990 to 2014 using a DEREP3 workflow (from oldest to most recent)

Natural sources and strategy	Goal	Ref
Screening of a collection of 116 marine sponges, ascidians, and cnidarians for their antifungal activity against *Candida albicans*	Extract prioritization	(Antonio and Molinski 1993)
Screening of a collection of aqueous extracts of terrestrial plants, cyanobacteria, and marine invertebrates for their AIDS-antiviral activity	Extract prioritization	(Cardellina et al. 1993)
Screening of 38,000 extracts from plants and microorganisms for antibacterial activity. Four promising extracts were retained after chemical dereplication	Early identification and elimination of known compounds	(Ramakrishn a et al. 1999)
LC/MS analysis of a plant extract library combined on-line to an estrogen receptor binding assay	Acceleration of bioactive compound characterization	(Schobel et al. 2001)
Curie-point pyrolysis MS and numerical taxonomic analysis of 44 deep-sea *Rhodococci*, clustering according to PyMS fingerprints and comparison with conventional microbial systematic classifications	Taxonomic classification	(Colquhoun et al. 2000)
Single quadrupole LC/MS discrimination of novel from known compounds in crude biologically active extracts	Early identification and elimination of known compounds	(Gilbert et al. 2003)
Screening of fungal metabolites and mycotoxins in cultured extracts using a LC/UV/MS micro-scale method and a MS-library	Early identification and elimination of known compounds	(Nielsen and Smedsgaard 2003)
Screening of 16 *Penicillium* species grown in different matrixes, and evaluation of produced metabolites *via* a chemo-diversity index	Evaluation of strain chemical diversity	(Nielsen et al. 2004)
Use of the ChemGPS database for a set of natural products exhibiting cyclooxygenase-1 and/or -2 (COX-1/2) inhibition	Investigation of correlations between structural features and bioactivity	(Larsson et al. 2005)
Screening of 456 bacterial isolates from marine sponges by using Intact-Cell MALDI-TOF-MS and proteometric clustering of the strains into 11 groups corresponding to particular species	Rapid selection of strains representing rare species for subsequent chemical characterization	(Dieckmann et al. 2005)
Analysis of four different collections of the sponge genus *Zyzzya* and comparative testing of these compounds in the National Cancer Institute's 60 human tumor cell lines	Search for anticancer compounds	(Dijoux et al. 2005)
Analysis of 500 bacterial isolates by GC/MS combined to HCA	Early identification of known compounds	(Boroczky et al. 2006)

Development of a simple method for high-throughput prefractionation of crude extracts	Removal of promiscuous inhibitors and interference compounds during enzyme/protein-based assays	(Appleton et al. 2007)
MALDI-TOF-imaging of intact marine cyanobacteria (*Lyngbya majuscula* 3L and JHB, *Oscillatoria nigro-viridis*, *Lyngbya bouillonii*, and *Phormidium* species) and sponges	Characterization of the spatial distribution of natural products	(Esquenazi et al. 2008)
Analysis of myxobacterial metabolites in nine *Myxococcus* species using LC/ESI-TOF combined to PCA	Investigation of potentially novel natural products, prioritization of candidates	(Krug et al. 2008)
Chemical profiling of fungal or bacterial extracts using an HPLC bioactivity profiling/microtiter plate technique in conjunction with capillary probe NMR instrumentation and databases	Demonstration of the discriminating power of H-1 NMR as a dereplication tool	(Lang et al. 2008)
Rapid determination of log P using UHPLC profiling gradients on a representative library of natural products and investigation of the relations log P– log k at different pH and UHPLC conditions	Implementation of dereplication database for crude plant extract profiling studies	(Eugster et al. 2009)
Monitoring of different substrate utilization, growth, secondary metabolite and antimicrobial profiles of some common filamentous fungal cultures using the Biolog FF MicroPlate, LC/MS and antimicrobial assays	Microbial drug discovery, dereplication of fungi and differentiation of closely related variants within one species	(Singh 2009)
Screening of aqueous and ethanol extracts of 5 South African medicinal plants for activity against HIV	Removal of non-specific undesired tannins and polysaccharides	(Klos et al. 2009)
NMR analysis of 30 New Zealand marine algal extracts, addition of all HSQC spectra to construct a "stacked HSQC digital mask" and substraction of this mask from each individual HSQC spectrum to enhance the unique signals	Extract prioritization	(Popplewell and Northcote 2009)
Metabolomic analysis of crude extracts obtained from actinomycetes in differents cultivation medium by direct infusion MS and LC/MS	Identification of novel microbial metabolites	(Crevelin et al. 2010)
Monitoring of aerial spore mass, pigment colours formed on oatmeal agar and capacity to produce melanin pigments of a range of alkaliphilic *Streptomycetes* isolated from a beach and dune sand system. Computer-assisted method for colour-grouping and comparison to PCR-data	Bioprospection and ecological study	(Antony-Babu et al. 2010)
LC/DAD/MS and NMR analysis of active crude extracts from *Abarema lusoria* and *Calea pinnatifida* and comparison with *in silico* databases	Understanding molecular relationships on dynamic natural matrixes	(Castro-Gamboa et al. 2010)
Explorative solid-phase extraction and ion-exchange chromatography for the chemical profiling of microbial extracts	Extract prioritization, mapping of biological activities	(Mansson et al. 2010)
Chemical screening of thirteen antibiotics and four cultivation broths by LC/DAD and fingerprint analysis based on polarity, UV spectra and acid-base properties	Identification of novel potent antibiotics among microbial metabolites	(Kamenik et al. 2010)

High-throughput LC/MS analysis of 16 025 microbial extracts, binning by nominal mass and retention time, data visualization by scattered plots and elimination of the ubiquitous peaks to focus on unique compounds	Investigation of extract chemical diversity	(Ito et al. 2011)
Analysis of 249 unidentified bacterial isolates retrieved from the rhizosphere of potato plants using MALDI-TOF MS, repetitive PCR or 16S rRNA gene sequence analysis and cluster analysis of the profiles obtained from the different techniques	Comparison of the taxonomic resolution obtained by different techniques for a broad diversity of bacteria	(Ghyselinck et al. 2011)
Analysis of fungal secondary metabolites in culture extracts using LC/DAD/MS with MS/MS spectral database	Search for novel potent anticancer compounds before engaging in isolation process	(El-Elimat et al. 2012)
Metabolomic analysis of marine-derived bacterial natural products using LC/MS with PCA	Strain prioritization in a drug discovery program	(Hou et al. 2012)
Molecular virtual design using NMR analysis of highly active crude extracts from Cerrado and Atlantic Rainforest specimens and endophytic fungi and microorganisms derived from specific rizhosphere habitats	Exploring Brazilian biodiversity	(Castro-Gamboa et al. 2012)
Chemical profiling of *Thymus vulgaris* extracts using LC/SPE/NMR and PCA to determine the discriminating constituents	Investigation of extract chemical diversity	(Pieri et al. 2009, Pieri et al. 2012)
Chemical profiling of 15 extracts of various organs of six *Lippia* species using LC/DAD/TOF-MS, MS spectral databases and HCA to highlight discriminating metabolites among species	Early identification of known compounds	(Eugster et al. 2011, Funari et al. 2012)
Chemical profiling of 15 extracts of six *Lippia* species by LC/DAD, comparison with standard compounds and PCA	Early identification of known compounds	(Funari et al. 2012)
Investigation of eight marine-derived *Furcatum* and *Penicillium* strains grown on six different culture media using LC/DAD/MS^n	Identification of novel potent bioactive compounds	(Vansteelandt et al. 2012)
Screening of non bioactive fungal extracts fermented in 8-medium nutritional arrays using LC/MS combined to an in-house database	Discovery of novel molecules with unknown activities	(Martin et al. 2012)
Chemical profiling of 22 phenolic extracts of extra virgin olive oil using LC/MS and *in vitro* cytotoxicity bioassay. Correlation analysis between the activity and LC/MS data and development of a model to predict the activity with further new samples	Rapid identification of bioactive compounds directly within crude extracts	(Roldan et al. 2013)
Metabolomic analysis of Streptomyces isolated from geographically varied environments using LC/MS combined to bucketing and presence-absence standardization strategy, PCA and HCA	Discrimination of microbial strains and identification of novel compounds	(Forner et al. 2013)
Dereplication of *Penicillium* strain extracts grown on various media using HPLC-UV/DAD-MS/MS	Assessment of toxigenic risks associated to fungal strains	(Geiger et al. 2013)

LC-MS-based metabolomic analysis of pure strain cultures and co-cultures of *Trichophyton rubrum* and *Bionectria ochroleuca*	Identification of novel metabolites induced by fungal interaction	(Bertrand et al. 2013)
Metabolomic analysis of 600 fungal co-cultures in solid media using UHPLC-TOF-MS, statistical comparison with the metabolite profiles obtained from mono-culture	Identification of novel metabolites induced by fungal interaction	(Bertrand et al. 2013)
Screening of filamentous fungi cultured extracts for the discovery of anticancer drug using LC/DAD/MS and MS/MS	Early elimination of extracts containing known compounds	(El-Elimat et al. 2013)
Chemical profiling of 22 African propolis samples using LC/UV/ELSD, LC/MS, GC/MS, LC/DAD/MS^2, heat mapping LC-UV and ELSD data, PCA and exact mass search in the DNP	Rapid identification of discriminating metabolites	(Zhang et al. 2014)
Morphological and chemical characterization of *Fusarium keratoplasticum* sp. and *Fusarium petroliphilum*	Chemical investigation of *Fusarium species*	(Short et al. 2013)
Characterization of the pigment profiles of 400 bacterial isolates using MALDI-TOF-MS dendrograms	Taxa discrimination and identification of novel carotenoids with UVA-Blue light absorbing properties	(Stafsnes et al. 2013)
Screening of a library of 3120 natural extracts with potent antibiotic properties, clustering of bioactivity profiles	Biological screening of novel potent antibiotics	(Wong et al. 2013)
Metabolomic analysis of 278 extracts from Malaysian biodiversity using LC/MS combined to PCA.	Prioritization of extracts potentially containing novel photosensitizers	(Samat et al. 2014)
Metabolomic analysis of antitrypanosomally active sponge-associated *Actinokineospora* sp. obtained in four fermentation conditions using HR-FT-MS and NMR combined to PCA, HCA and OPLS-DA	Identification of the best culture one-strain-many-compounds conditions	(Abdelmohsen 2014)
Chemical profiling of 77 bacterial extracts isolated from cold water marine invertebrates from Scotland using LC/MS combined to PCA and NMR (^{1}H and COSY)	Acceleration of strain prioritization	(Macintyre et al. 2014)
Dereplication of *Aspergillus carbonarius* and *Penicillium melanoconidium* extracts using LC/DAD/ESI-MS, overlay of automatically generated extracted-ion chromatograms to visualize novel peaks	Screening of novel bioactive metabolites	(Klitgaard et al. 2014)

Dereplication and targeted chemical profiling: two terms coming down to the same approach (DEREP4)

As mentioned above, in a targeted metabolite profiling approach a predefined number of compounds or a particular chemical class of compounds is investigated and the molecular structures are identified. Remembering that the literal definition of dereplication is "*the rapid*

identification of known compounds", and assuming that the range of compounds investigated in targeted chemical profiling studies can also be known, it can be considered that both concepts are quite similar in many cases and that a targeted chemical profiling is necessarily a kind of dereplication, even if in contrast dereplication does not always correspond to a targeted chemical profiling approach.

In the literature survey, 57 studies (18 % of the total examined articles) involving dereplication were used a targeted chemical profiling approach. These studies are summarized in Table 5 and are noted as DEREP4 strategies. Their global workflow is given in Fig. 5.
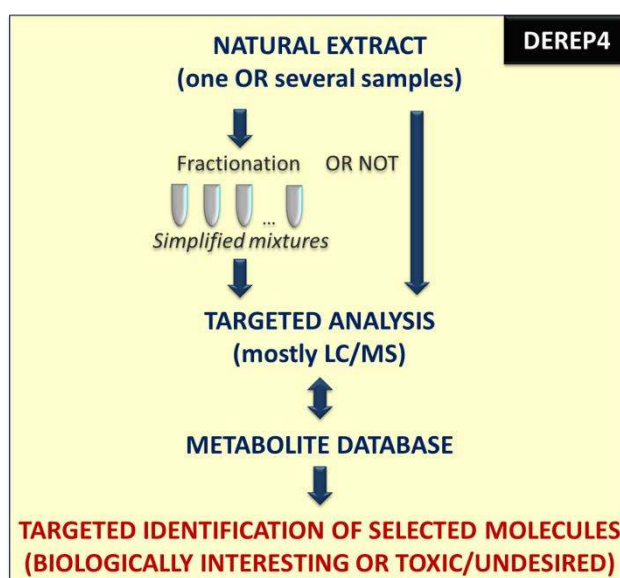

Fig. 5. DEREP4: a targeted dereplication workflow focused on the identification of a predefined group of known molecules

The main objective of DEREP4-based studies is either to analyze a particular group of molecules presenting a biological interest or on the contrary to identify potentially toxic or undesired constituents of crude natural extracts. For example, the dereplication of free sugars and polyols was performed by GC/MS in a study investigating novel natural sweeteners. Among the sweet-tasting species analysed, the extracts containing high levels of these

compounds were regarded as lower priority leads and thus directly removed from further consideration, enabling a significant time gain (Chung et al. 1997). DEREP4 approaches are also commonly used to assess the quality of botanical drugs that must be checked for authentication, contamination, and identification of active substances or safety validation. Such procedures are based on the fact that all natural extracts, although highly complex, contain secondary metabolites which are specific to the species under study. During analytical data capture, the set of signals corresponding to these specific metabolites becomes a chemical fingerprint and the most characteristic signals are called "chemical markers". For instance three major concentrated stilbenoids quickly identified by a HPTLC/MS-based dereplication approach and exhibiting skin-anti-ageing properties were reported as relevant markers of a range of orchid species (Williams et al. 2012). Of course, concentration variability of these chemical markers in natural raw materials is inevitable due to inherent factors such as genotype variability, growing conditions, and climate or soil type. By tracking chemical markers over different steps of the manufacturing processes, the DEREP4 approach can assess this variability and minimize the potential composition heterogeneity of the final ingredient from one batch to another. This procedure forms an integral part of the standardization of botanical drugs, a major task which is necessary to adjust the concentration of the active substances and ensure a reproducible quality of the final products.

In the vast majority of targeted chemical profiling studies, natural metabolites were analyzed by mass spectrometry. This can be explained by the fact that a group of targeted compounds generally comprises structurally related molecules, consequently exhibiting very similar fragmentation patterns. The use of MS-based libraries containing fragmentation spectra and substructure data of the targeted compounds is thus very useful in the case of such dereplication approaches. For instance, a range of taxoids were successfully identified in a crude extract of *Taxus wallichiana* with the help of a MS library containing fragmentation spectra of 3 taxoid

standards and substructure spectra of 139 previously reported toxoids (Stefanowicz et al. 2001). Similarly, it was found that retro-Diels-Alder rearrangement occurring during the MS fragmentation of a xanthone skeleton produce characteristic fragment ions which could be used to target prenylated xanthones in plant extracts such as *Garcinia* plants (Zhou et al. 2008). The fragmentation pattern of flavonoids and their glycoside derivatives is also commonly used to dereplicate this class of compounds in crude natural extracts through targeted chemical profiling approaches (Constant et al. 1997, Waridel et al. 2001, Tchoumtchoua et al. 2013). Mass accuracy and isotopic patterns are other data recovered from MS and MS[n] acquisition that can be used to build up libraries of natural metabolites for dereplication purposes (Gomez-Romero et al. 2011).

It can be supposed that NMR-based metabolite profiling strategies are less efficient to perform the targeted chemical profiling of a predefined set of compounds, mainly because of the strong NMR signal overlaps observed when analysing a group of structurally related molecules. However, several studies have demonstrated that this issue can be overcome by focusing on specific NMR signals of the targeted molecules. The screening of several brominated tyrosine derivatives named bastadins in a crude extract of the marine sponge *Lanthella basta* was performed for instance by ^{1}H NMR detection of their characteristic methoxy signals (Franklin et al. 1996). In the same way, diagnostic ^{1}H NMR signals of epoxide and conjugated diene moieties have been used to dereplicate macrocyclic trichothecenes in filamentous fungal species in the context of new anticancer lead discovery (Sy-Cordero et al. 2010).

Finally, it should be mentioned that targeted chemical profiling approaches not only enable the identification of previously known compounds, but also promote the discovery of additional novel metabolites, often corresponding to structural analogs of the predefined class of targeted compounds.

Table 5. Dereplication strategies published from 1990 to 2014 using a DEREP4 workflow (from oldest to most recent)

Targeted compounds and natural sources	Analytical technique(s)	Ref
Cucurbitacins in *Iberis amara*, *Begonia plebeja* and *Gonystylus keithii*	Computer-Assisted recognition of cytotoxicity profiles	(Fuller et al. 1994)
Elaiophylin and geldanamycin in a range of microbial broths	CPC/DAD, LC-MS	(Alvi et al. 1995)
Two known antibiotics teicoplanin and phenelfamycin in several microbial crude extracts	LC/UV/ESI-MS/MALDI-MS	(Ackermann et al. 1996)
Bastadins from *Lanthella basta*	^{1}H NMR	(Franklin et al. 1996)
Free sugars and polyols in six sweet-tasting plant extracts	GC/MS	(Chung et al. 1997)
Flavonoids and flavonoid glycosides using an aqueous extract of *Eugenia jambos* as a model	LC/MS and CID for fragmentation patterns	(Constant and Beecher 1995, Constant et al. 1997)
Potentially interfering polyphenols during the biological screening on *in vitro* cytotoxicity bioassays of 3000 plant extracts from tropical rainforests against a panel of human cancer cell lines.	LC/UV/MS	(Kinghorn et al. 1995, Cordell and Shin 1999, Kinghorn et al. 1999)
Seven destruxins in the fungus *Metarrhizium anisopliae*	HPLC/ESI-TOF-MS	(Potterat et al. 2000)
Taxoids from *Taxus wallichiana*	MS^n	(Stefanowicz et al. 2001)
C-glycosidic flavonoids	LC/MS/MS using Q-TOF and IT under various CID energy conditions	(Waridel et al. 2001)
Sesquiterpenes from two Indo Pacific sponges *Lendenfeldia frondosa* and *Hyrtios* sp.	NMR	(Stessman et al. 2002)
Trichothecenes from seven microbial *Trichoderma* species grown on several solid and liquid media	LC/UV/vis and ESI-MS	(Nielsen et al. 2005)
Betaxanthins from yellow-orange cactus pear fruits and yellow Swiss chard petioles	^{13}C NMR	(Stintzing et al. 2006)
High molecular weight polysaccharides from several mushroom extracts	DOSY-based NMR	(Politi et al. 2006)
Pentacyclic triterpenoids as their methyl esters (oleanolic acid, betulinic acid and ursolic acid) with potent anti-tubercular activity	GC/ESI-MS	(Gu et al. 2006)
Saponins and acyclic sesquiterpene oligoglycosides in the fruits of *Sapindus saponaria*	LC/UV/ESI-MS, MS/MS	(Murgu and Rodrigues 2006)
Anti-inflammatory compound vicenin-2 in *Leucopogon ericoides*	LC/ESI-MS^n	(de Moraes et al. 2007)

(+)-psymberin and a new brominated cyclic peptide (-)-psymbamide A from the sponge *Psammocinia aff. bulbosa*	ESI-MS, NMR	(Robinson et al. 2007)
Novel phenolic compactin analogue from *Penicillium solitum*	UV-guided strategy, NMR, MS	(Larsen et al. 2007)
Valerenic acid in a valerian extract and other GABA(A) receptor ligands in plant and fungal extracts	HPLC	(Kim et al. 2008)
Streptothricin-like compounds in the fermentation broth of *Streptomyces qinlingensis*	Ion-pair LC/ESI-MS	(Ji et al. 2008)
Polyprenylated xanthones in *Garcinia xipshuanbannaensis*	On-line LC/ESI-QTOF-MS[3]	(Zhou et al. 2008)
Alkaloids in several *Colchicum* species	LC/MS, LC/UV/DAD	(Alali et al. 2008, Gharaibeh et al. 2012)
Secoiridoid glycosides in *Sarracenia alata*	capillary-scale NMR, ESI-MS	(Hu et al. 2009)
Plumeran indole alkaloids in a stem bark extract of *Aspidosperma spniceanum*	ESI-MS/MS	(Aguiar et al. 2010)
Bufadienolides in the TCM toad skin	UHPLC/ESI-Q-TOFMS	(Liu et al. 2010)
Bromotyrosine-derived metabolites in 14 specimens of *Aplysina* spp. marine sponges	UV, LC/DAD/MS	(Silva et al. 2010)
Heterocyclic nerolidol derivatives from the anti-tuberculosis active fractions of the inner stem bark of *Oplopanax horridus*	^{1}H NMR with focus on spin-spin coupling patterns	(Inui et al. 2010)
Macrocyclic trichothecenes from different fungal species	UV spectra, ^{1}H NMR	(Sy-Cordero et al. 2010)
Bastadins from the marine sponge *Ianthella* reticulate	NMR	(Calcul et al. 2010)
Phenolics in propolis and lyophilisate of some vegetables selected for their antioxidant properties	LC/ESI-QTOF	(Gomez-Romero et al. 2011)
Stilbenoids in several species of the Orchidaceae family	MicroCryoProbe NMR	(Williams et al. 2012)
Pregnane glycosides and genins from the TCM *Marsdenia tenacissima*	HPLC/ESI-MS[n]	(McGarvey et al. 2012)
Aporphine and oxoaporphine alkaloids from *Unonopsis guatterioides*	direct infusion in ESI-IT-MS	(da Silva et al. 2012)
Active photosensitisers possessing a cyclic tetrapyrrole in 15 plant extracts	LC-DAD-MS	(Tan et al. 2012)
Nonribosomal peptides	MS/MS, chemoinformatic library	(Ibrahim et al. 2012)
Bisbenzylisoquinoline alkaloids from the fruit of *Gyrocarpus jacquinii*	UV, MS	(Klausmeyer et al. 2012)
Annonaceous acetogenins from *Annona muricata*	LC/LTQ-Orbitrap	(Le Ven et al. 2012)
170 known triterpenes from *Actaea* species	^{1}H NMR and predictive model using classification binary trees	(Qiu et al. 2012)

Aliphatic alkaloids from *Huperzia selago* and bisbenzylisoquinoline alkaloids from *Triclisa patens*	LC/SPE/NMR	(Johansen et al. 2012).
Minor cytotoxic chlorinated valepotriates from whole plants of *Valeriana jatamansi*	HPLC-PDA-MS, TLC, NMR	(Lin et al. 2013)
Steroidal alkaloids in complex plant extracts of *Buxus* species	ESI-TOF-MS/MS	(Musharraf et al. 2013)
Thiazolyl peptides from	Miniaturized 96-well SPE , HR-FT LC/MS and library	(Singh et al. 2013)
Lasiodiplodins from a cytotoxic extract obtained from *Lasiodiplodia theobromae*, an endophyte from the root tissues of *Mapania kurzii*	ESI-MS, NMR	(Sultan et al. 2014)
Ubiquitous flavonoids and related plant constituents	Q-TOF, IT and MS^2 library	(Wolfender et al. 2000)
Rhizoxins and rhizonins in different Zygomycetes grown on a range of semisynthetic and natural substrates	LC/DAD/HR-MS	(Jennessen et al. 2005)
Stilbenoids in wine fractions	LC/DAD/MS, LC/NMR	(Pawlus et al. 2013)
Flavonoids and benzophenone derivatives from *Qualea grandiflora* and *Qualea cordata* extracts	HPLC/ESI-QTOF-MS/MS	(Neto et al. 2013)
Isoflavonoids from a stem bark of *Amphimas pterocarpoides*	UHPLC/MS, MS/MS	(Tchoumtchoua et al. 2013)
Pyrrolamide compounds including congocidine and distamycin in fermentation culture of *Streptomyces netropsis*	genome scanning and precursor ion scan MS	(Hao et al. 2014)
Triterpenes from various *Actaea* species	2D-NMR barcoding	(Qiu et al. 2014)
Trace-levels of acetogenins in an *Annona cherimolia* fruit-based alcoholic beverage	LC/HRMS with postcolumn infusion of lithium iodide	(Le Ven et al. 2014)
Surfactin-type lipopeptides in *Bacillus* cultures from commercial Japanese foods	LC/HRMS	(Juola et al. 2014)

Molecular genetics and taxonomic dereplication of microbial strains (DEREP5)

Microbial natural products are a major resource for the development of novel drugs in pharmaceutical industry. However, the chemical characterization of microbial secondary metabolites is currently hampered by the laborious isolation processes when working with culture broths, and by the limited availability of structural and spectral libraries for microbial

metabolites. In addition, microbial culture collections are extremely diverse, and thus a major challenge in their rational exploitation is the reduction of unnecessary or redundant testing of strains. In this sense, a range of dereplication tools mainly based on phenotypic characteristics or genetic differences have been developed to select microbial isolates at the species level. Genetics and metabolic engineering are currently an integral part of these dereplication strategies. In recent years, the exploration of new taxa or ecological niches through metagenomic approaches have provided access to a large number of gene sequences that can now be investigated not only in well-studied bacteria but also in microorganisms that were previously neglected as natural ressources. The metagenome can be defined as a "collective genome" including isolated microbial DNA of all microorganisms present in a particular habitat (Handelsman et al. 1998). "DEREP5" will thus denote all studies (n=22, 7 % of the total examined articles) found in the literature survey that involved a genetic approach for the dereplication of microbial strains. These studies are all included in Table 6 and their workflow is illustrated in Fig. 6.
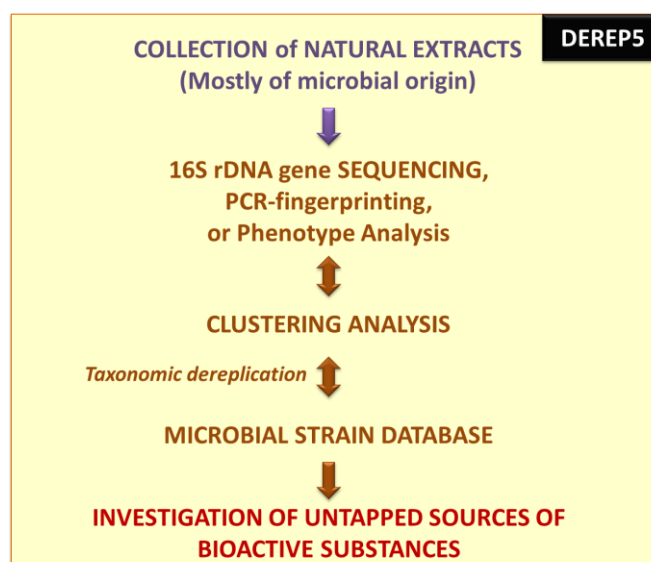

Fig. 6. DEREP5: A taxonomic dereplication workflow based on gene-sequence analyses of collection of microbial strains

It must be specified that many DEREP5 studies were performed on microbial strains or isolates "as a whole" and did not systematically include a detailed analysis of the metabolites produced by the selected microorganisms. Nevertheless, such approaches have already led to the construction of extensive taxonomy-guided microbial natural product libraries, and thus enables for instance biodiversity surveys, strain prioritization, spatial distribution characterization, biosynthetic pathway elucidation, discovery of untapped sources of natural products, and detection of uncommon genera or species among microbes. Most of the current protocols for the taxonomic dereplication of microbial strains involve 16S rDNA gene sequencing or PCR-fingerprinting of large collections of isolates followed by clustering analysis in order to correlate phylogenic similarities or to highlight discriminating strains. Once microorganisms have been taxonomically identified and revealed as promising leads, more sensitive analytical tools can be implemented to investigate metabolite profiles and potentially identify novel bioactive compounds. Strategies for *in silico*-guided identification of secondary metabolites by genome mining have been highlighted in several reviews (Zerikly 2009, Helfrich et al. 2014, Helfrich 2014). Such strategies seem promising, but although novel compounds have been revealed in activity-driven screening of gene libraries, the frequency of the identified compounds is relatively low (Novakova and Farkasovsky 2013). Research efforts are currently being reinforced to better understand microbial and chemical ecology, improve microbial culture conditions, high-throughput cultivation and performance of large-scale biological screening assays (Ashforth et al. 2010, Baltz 2010, Penesyan et al. 2010). Improvements in sequencing capabilities would also contribute to this area, particularly regarding primer design, increase of insert sizes and diversification of library hosts (alternative to *Escherichia coli*-based libraries).

It must finally be mentioned that the large majority of DEREP5 studies are aimed at screening novel antibacterial metabolites. Pathogen resistance to currently available antibiotics has indeed

reached a very critical level. Over the last twenty years, most pharmaceutical companies have abandoned natural product chemical profiling investments in favor of large and cost-effective libraries of synthetic molecules. However, it is obvious that further improvements in genome sequencing technologies, together with the use of modern analytical systems, computer tools and application of reliable and pertinent biological assays will progressively solve the speed-limiting dereplication obstacles and lead these companies to refocus on natural product candidates (Kirst 2013). Several reviews addressing the value and uses of molecular genetics-based dereplication procedures applied to natural microbial metabolites are available in the literature (Strobel 2002, Knight et al. 2003, Leeds et al. 2006, Liu et al. 2010, Genilloud et al. 2011, Roemer 2011, Genilloud 2014, Sandiford 2014).

Table 6. Molecular genetic-based strategies developed for the taxonomic dereplication of microbial natural products (DEREP5)

Strategy	General goal	Ref
Investigation of unusual strains of actinomycetes and filamentous fungi by reconstruction of gene clusters from small segment of cloned DNA and preparation of large-insert libraries	Detection of uncommon genera of soil microbes	(Donadio et al. 2002)
Identification of 26 closely related *Streptomyces* soil isolates using rDNA sequencing, MIDI fatty acid analysis, and LC-MS profiling of fermentation extract	Discrimination between morphologically similar strains	(Ritacco et al. 2003)
Screening of 20 *Micromonospora* isolates from UK coastal sediments, taxonomic classification by rDNA sequencing, fluorescent amplified fragment length polymorphism (AFLP), and Fourier transform infrared spectroscopy (FT-IR)	Investigation of strain diversity	(Zhao et al. 2004)
Targeted analysis of polyketide synthases and nonribosomal peptide synthetases amplified sequences in a collection of wild-type Actinomycetes isolated from tropical soil samples evaluated for the production of antimicrobial activities	Characterization approach for Actinomycetes	(Ayuso et al. 2005)
Dereplication of microorganisms collected in 12 aquaculture sites in Southeast Asian and using PCR fingerprinting	Investigation of biodiversity and environmental distribution of chloramphenicol-resistant (CmR) mesophilic heterotrophs	(Huys et al. 2007)

Evaluation of the antimicrobial activity and sub-grouping of 217 streptomycetes isolates from the water surface microlayer in Norway by phylogenetic analysis and 16S rDNAs sequencing. 7 isolates with identical 16S rDNA sequences were further studied for the presence of PKS type I genes	Investigation of Actinomycetes from the water surface microlayer as a source of new antimicrobial agents	(Hakvag et al. 2008)
RNase P RNA gene (rnpB) sequencing of 50 myxobacteria strains for the faster and cheaper discrimination of similar strains as compared to 16S rDNA sequencing	Development of RNase P RNA gene (rnpB) sequencing as a tool for molecular dereplication	(Monciardini et al. 2008)
Isolation of *Micromonospora* strains on the basis of typical colonial and pigmentation features and 16S rRNA gene sequencing analyses and correlation with molecular fingerprinting	Taxonomical classification	(Maldonado et al. 2008)
Dereplication of 127 *lactobacilli* isolates by (GTG)(5)-PCR fingerprinting, phylogenetic analysis of the 51 resulting genotypes using a combined amplified 16S rDNA restriction analysis (16S-ARDRA), species-specific PCR assays and 16S rRNA gene sequencing	Investigation of lactic acid bacteria strain diversity in 31 ripened Parmigiano Reggiano cheeses	(Solieri et al. 2012)
ITS1 and ITS4-rDNA sequencing of four endophytic fungi extracts evaluated in parallel for their cytotoxic activity, identification of known compounds using an UV library	Investigation of new potent cytotoxic fungal extracts	(Hazalin et al. 2012)
Multigene analysis, combined with phenotypic characters and extrolite profile of *Penicillium* sect. *Chrysogena* species	Taxonomic classification of penicillin-producing strains	(Houbraken et al. 2012)
Development of a web-based bioinformatics platform (FastGroupII) to dereplicate large 16S rDNA libraries and application on a set of 16S rDNA sequences from coral-associated bacteria	Development of bioinformatic tools for the analysis of high-throughput 16S rDNA sequencing databases	(Yu et al. 2006)
Automated ribotyping of 157 isolates following restriction enzyme digestion, classification into 23 ribogroups representing a 85% reduction of the number of isolates in the library	Dereplication of a large collection of phenotypically ambiguous bacterial isolates	(Sheffield et al. 2006)
16S rRNA, gyrB and recA gene sequencing of thirteen *P. luteoviolacea* strains to determine whether chemotype and activity profile can be reflected by phylogenetic clustering	Selection of strains for antibiotic discovery	(Vynne et al. 2012)
Dereplication of 433 Danish surface-ripened cheeses bacterial isolates using (GTG)5-PCR fingerprinting, and identification of 217 bacterial and 25 yeast isolates by 16S rRNA gene sequencing	Phylogenetic analysis of bacterial diversity in Danish cheeses	(Gori et al. 2013)
Characterization of microbial eukaryotic populations associated with Pyrenean glaciers using molecular 18S rRNA-based approaches, amplifying community DNA and constructing clone libraries with 18S rRNA primers	Phylogenetic analysis of microbial eukaryote diversity in icy regions	(Garcia-Descalzo et al. 2013)
Taxonomic identification and phylogenetic reconstruction of 138 cellular extracts of wheat-associated bacterial	Assessment of bacterial isolate diversity	(Stets et al. 2013)

isolates using 16S rRNA gene sequencing and whole cell MALDI-TOF-MS analysis		
DNA expression array analysis of Aspergillus nidulans in combination with legacy data to form a comprehensive gene expression compendium and data clustering to identify cross-chemistry between physically separate gene clusters	Elucidation of biosynthetic pathways of fungal secondary metabolites	(Andersen et al. 2013)
Development of a two-pronged approach for the characterization of inhibitors of protein synthesis (ChIPS) by engineering antibiotic-hypersensitive Escherichia coli strains that contain only one rRNA operon and elucidation of the mode of antibiotic action by monitoring drug-induced ribosome stalling on mRNA	Rapid identification of the site and mode of action on the bacterial ribosome	(Orelle et al. 2013)
PCR analysis of 100 strains randomly selected from an actinomycete collection to evaluate their ability to biosynthesize aromatic polyketides, reduced polyketides, nonribosomal peptides, and diterpenoids	Extract prioritization by biosynthetic potential survey	(Xie et al. 2014)
Investigation of a collection of *Aspergillus nomius* strains from Brazil nuts including morphological characters, RAPD and AFLP profiles, partial β-tubulin and calmodulin nucleotide sequences, aflatoxin patterns, as well as tolerance to low water activity in cultured media	Characterization of *Aspergillus* species in Brazil nuts	(Massi et al. 2014)
Investigation of *Streptomyces lavendulae* phenotypic cluster for production of lipstatin-like lipase inhibitors using a taxonomy-based dereplication with a public collections of strains and *in vitro* assays	Identification of potentially novel and useful industrial *Streptomyces* strains	(Sladic et al. 2014)

Conclusion

Dereplication procedures now play a prominant role in the field of natural product research. The examination of all bibliographic references published from 1990 to 2014 which involve the concept of dereplication clearly reveals that five different dereplication strategies have been gradually developed to meet specific objectives. The common denominator between these studies is the willingness to accelerate the discovery of biologically active substances by improving characterization methods of natural resources. Most dereplication workflows have addressed this issue by using targeted or untargeted chemical profiling strategies, often in combination with biological assays and computer tools to assess the presence of active

metabolites in natural samples, while others have used a taxonomic approach mainly based on genetic analyses for the classification and prioritization of samples. It is quite clear that the quality and completeness of small metabolite databases and microbial strain libraries remain the major impediments to high performance dereplication workflows. Consequently, the author can only stress the importance of finding a way to link collected spectral data between laboratories and finally homogenize these databases.

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